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Defective liver formation and liver cell apoptosis in mice lacking the stress signaling kinase SEK1/MKK4

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SUMMARY

The stress signaling kinase SEK1/MKK4 is a direct activator of stress-activated protein kinases (SAPKs; also called Jun-N-terminal kinases, JNKs) in response to a variety of cellular stresses, such as changes in osmolarity, metabolic poisons, DNA damage, heat shock or inflammatory cytokines. We have disrupted the *sek1* gene in mice using homologous recombination. *Sek1*$^{-/-}$ embryos display severe anemia and die between embryonic day 10.5 (E10.5) and E12.5. Haematopoiesis from yolk sac precursors and vasculogenesis are normal in *sek1*$^{-/-}$ embryos. However, hepatogenesis and liver formation were severely impaired in the mutant embryos and E11.5 and E12.5 *sek1*$^{-/-}$ embryos had greatly reduced numbers of parenchymal hepatocytes. Whereas formation of the primordial liver from the visceral endoderm appeared normal, *sek1*$^{-/-}$ liver cells underwent massive apoptosis. These results provide the first genetic link between stress-responsive kinases and organogenesis in mammals and indicate that SEK1 provides a crucial and specific survival signal for hepatocytes.

Key words: SEK1/MKK4, Stress kinase signaling, Hepatogenesis, Mouse

INTRODUCTION

During the development of all multicellular organisms, cell fate decisions determine whether cells undergo proliferation and differentiation, or apoptosis. Distinct and evolutionarily conserved signal transduction cascades mediate survival or death in response to developmental programs and environmental triggers (Cobb and Goldsmith, 1995; Treisman, 1996; Woodgett et al., 1996). Stress-activated protein kinases (SAPK/JNK) are activated in response to many developmental and environmental cues, including cellular stresses such as metabolic poisons, anti-cancer drugs, DNA damage, irradiation, inflammatory cytokines, heat shock and ischemia. SAPK activity is also induced in response to growth factors, heterotrimeric G-proteins, phorbol esters or costimulation of T cells (Derijard et al., 1994; Kyriakis et al., 1994; Minden et al., 1994a; Prasad et al., 1995; Raingeaud et al., 1995; Su et al., 1994; Westwick et al., 1995). When activated, SAPKs translocate from the cytoplasm to the nucleus and phosphorylate transcription factors such as c-Jun (Angel and Karin, 1991).

SAPKs are activated by the phosphorylation of tyrosine and threonine residues in a reaction that is catalyzed by the dual specificity kinases SEK1 (also known as MKK4 and JNKK1) (Derijard et al., 1995; Lin et al., 1995; Sanchez et al., 1994; Yan et al., 1994) and SEK2 (MKK7, JNKK2) (Holland et al., 1997; Lu et al., 1997; Tournier et al., 1997; Toyoshima et al., 1997; Wu et al., 1997; D. Yang et al., 1997a; Yao et al., 1997). SEK1-dependent and SEK1-independent pathways for SAPK activation operate in response to specific stresses, and different types of stimuli trigger distinct signaling pathways for SAPK activation (Nishina et al., 1997b; D. Yang et al., 1997b). Moreover, induction of these two signaling cascades is developmentally regulated in T cells (Nishina et al., 1997a). Thus, cells have developed two parallel signaling pathways for the activation of SAPKs. These signaling pathways are mediated by SEK1/MKK4 and SEK2/MKK7 and may have evolved to sense different types of environmental stress and developmental stimuli.

In *Drosophila*, the gene products of *basket (bsk)* and *hemipterous* (*hep*) regulate epithelial sheet movement and

dorsal closure during fly morphogenesis (Glise et al., 1995). *Basket* is the fly homologue of mammalian SAPK/JNK and *hep* was originally identified as a homologue of SEK1. However, *sek2/mkk7* has greater sequence homology to *hep* and SEK2 is highly expressed in epithelial tissues (Yao et al., 1997). Moreover, mammalian SEK2 can functionally complement the *hep* defect, indicating that SEK2 is the mammalian *hep* homologue (Holland et al., 1997). *Drosophila* embryos lacking the c-Jun homologue *djun* also exhibit a defect in dorsal closure (Kockel et al., 1997), suggesting that the SEK→SAPK→Jun signaling cascade has a role in the same developmental processes. However, DJun-dependent cell fate specification in the eye does not require Hep or Bsk (Kockel et al., 1997; Riesgo-Escovar et al., 1996), implying that DJun plays a crucial role in other developmental signaling pathways during development. In mice, *c-jun*$^{-/-}$ embryos display defective liver organization and die between E13.5 and E14.5 (Hilberg et al., 1993; Johnson et al., 1993). The biological roles of mammalian SEK1 and SEK2 and SEK-regulated SAPK/JNK activation in mammalian development and morphogenesis are not yet known.

To elucidate the biological function of SEK1, we generated two different lines of *sek1* null mice using homologous recombination. These *sek1*$^{-/-}$ embryos die between day 10.5 and 12.5 of gestation. The mutant embryos are severely anemic but display normal vasculogenesis and normal haematopoiesis of erythroid, lymphoid and myeloid cells from yolk sac precursors. Surprisingly, *sek1*$^{-/-}$ embryos have a severe and specific defect in liver formation and hepatocytes development. *Sek1*$^{-/-}$ embryos can form the primordial liver anlage but liver cells undergo massive apoptosis.

MATERIALS AND METHODS

Sek1 gene targeting

Sek1 gene targeting and the *sek1* targeting construct have been described previously (Nishina et al., 1997). Briefly, an 8 kb genomic SEK1 fragment was isolated from a genomic 129/J library. Twelve nucleotides within exon 2 of the SEK1 gene were replaced by a *neomycin* (Neo) gene cassette containing a poly(A) termination signal inserted in sense orientation. The linearized construct was introduced into E14K ES cells by electroporation. G418-resistant ES colonies were screened for homologous recombination by PCR and Southern blotting. The frequency of homologous recombination was about 1 in 800. For the generation of SEK1$^{-/-}$ ES cell lines, G418-resistant SEK1$^{+/-}$ ES clones were cultured at increasing concentrations of G418 (2 mg/ml). For the generation of null mice, two independent *sek1*$^{+/-}$ ES cell lines were injected into C57BL/6 blastocysts. Chimeras were further bred with C57BL/6 female mice. Germline transmission was achieved with both *sek1*$^{+/-}$ ES cell lines. The absence of SEK1 was confirmed by northern blotting of 10 μg of poly(A)-selected RNA using a full-length SEK1 probe. SEK1 protein levels were determined by immunoblotting using a rabbit-anti-SEK1 Ab reactive against the kinase domain of SEK1 (Upstate Biotechnology).

Mice

C-jun-deficient (Johnson et al., 1993) and *c-fos*-deficient (Grigoriadis et al., 1994) mice were purchased from the Jackson Laboratory Induced Mutant Depository. *Flk1-lacZ* knock-in mutant mice have been described previously (Yamaguchi et al., 1993) and were a kind gift of Dr Janet Rossant, Mount Sinai Hospital, Toronto. *Flk1-LacZ sek1* and *c-jun sek1* double mutant mice were generated by interbreeding of mice heterozygous for the respective mutations. Mouse genotypes were determined by PCR. The following PCR primer pairs were used:

Sek1 mutation: sense primer 5′-ACAGCAAATTTTGGAAAC-AGC-3′ and antisense primer 5′CTCCCCTACCCGGTAGAATTC-3′;

Sek1 wild type: sense primer 5′-ACAGCAAATTTTGGA-AACAGC-3′ and antisense primer 5′-AATTTCTCCAAGG-TCTTTCAA-3′;

Flk1 mutation: sense primer 5′-CTGTGTCCCGCAGCCGGATA-3′ and antisense primer 5′-AAAGCGCCATTCGCCATTCA-3′;

Flk1 wild type: sense 5′-CTGTGTCCCGCAGCCGGATA-3′ and antisense primer 5′-AAGTCACAGAGGCGGTATGG-3′;

c-Jun mutation: sense 5′-AGCGGTTCCTTGGAGCCCGC-3′ and antisense primer 5′-TAAAACGCACGGGTGTTGGGT-3′;

c-Jun wild type: sense 5′-GCTAGCACTCACGTTGGTAGG-3′ and antisense primer 5′-CTTCCACCGAGAATTCCGTGA-3′.

For timed pregnancies, male and female mice were mated overnight and female mice were scored for vaginal plaques the next morning. The presence of vaginal plaques was taken to represent embryonic day 0.5 (E0.5). All mice were kept in the animal facility of the Ontario Cancer Institute in accordance with institutional guidelines and those of the Canadian Medical Research Council.

Western blotting

ES cells (1×10^6) and cell suspensions (1×10^6 cells) of whole embryos were lysed in 0.5% NP-40 lysis buffer. Total proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto PVDF membranes (Dupont). The blots were then probed with polyclonal rabbit anti-SAPK/JNK IgG reactive against all SAPK/JNK isoforms (Nishina et al., 1997), rabbit-anti-SEK1 Ab (New England Biolabs) and rabbit anti-c-Jun Ab (New England Biolabs). Primary Ab binding was detected using horseradish peroxidase-labelled swine anti-rabbit Ig (Dako) and antibody binding was visualised using the enhanced chemiluminescence (ECL) detection system (Amersham Life Science Inc.).

Histology

Embryos were isolated in ice-cold PBS at E7.5, E8.5, E9.5, E10.5, E11.5, E12.5, E13.5 and E14.5, fixed overnight in 4% paraformaldehyde at 4°C, dehydrated and embedded in paraffin. 6 μm-thick sections were cut and stained with hematoxylin and eosin (Bachmaier et al., 1997).

Immunohistochemistry

Whole E10.5 and E12.5 embryos were processed for cryosections. For immunoperoxidase staining, cryostat sections were fixed in acetone and the endogenous peroxidase activity was blocked using 0.6% NaN_3 and 0.125% H_2O_2 (Bachmaier et al., 1997). Sections were incubated with the following monoclonal antibodies: mouse anti-PCNA to detect proliferation (Tago), rat anti-Cytokeratin (Sigma) to detect parenchymal hepatocytes, rat-anti-CD45 (Pharmingen) to detect hematopoietic cells, and rat anti-TER119 (Pharmingen) to detect erythroid precursors. Binding of primary rat and mouse Abs was detected using peroxidase-conjugated rabbit anti-rat IgG or rabbit-anti-mouse IgG antibodies (dilution 1:100; Dako, #P-162). Antibody binding was visualized by conversion of substrates (Sigma Fast DAB, #D-0426). Sections were counterstained with hematoxylin.

Immunocytochemistry and X-Gal staining in whole mouse embryos

For whole-mount immunostaining, embryos were collected in PBS, the extraembryonic membranes were dissected away and the embryos were fixed in methanol:DMSO (4:1, overnight at 4°C) followed by processing for whole-mount immunostaining as described (Marti et al., 1995). Whole embryos were incubated with a primary rabbit anti-HNF3β antiserum (1:1000 dilution, 4°C overnight) and binding of the

primary Ab visualised by a horseradish peroxidase-labelled secondary goat anti-rabbit antiserum (Sasaki et al., 1997). Staining was developed using Sigma-FAST-DAB as a substrate. Whole-mount X-Gal staining for *flk1*-driven β-galactosidase activity performed as described (Fong et al., 1995). Briefly, embryos were fixed in 4% paraformaldehyde and stained for β-galactosidase activity using X-Gal staining solution (Life Technologies) for 3 hours at 32°C.

Apoptosis detection in situ

E10.5 and E12.5 embryos were snap frozen in liquid nitrogen and processed for immunostaining**.** TUNEL reactions to detect incorporation of dioxygenin-dUTP mediated by terminal transferase were performed on sectioned embryos using a commercial kit (ApopTag in situ apoptosis detection Kit – peroxidase; Oncor Gaithersburg, MD). TUNEL reactions were visualised using a horseradish peroxidase-labelled anti-dioxigenin Ab and Sigma-FAST-DAB as a substrate (see above for method and blocking of endogenous peroxidase activity).

In situ hybridization

Uteri were isolated in ice-cold phosphate-buffered saline at E10.5, E12.5, E14.5, E16.5 and E18.5, and embryos were processed for histological analysis. SEK1 (MKK4) antisense and SEK2 (MKK7) antisense probes, and control SEK1 (MKK4) sense and SEK2 (MKK7) sense probes, were prepared using in vitro transcription (Yao et al., 1997). Embryonic sections were processed, hybridized and developed as described (Hui and Joyner, 1993).

Methylcellulose colony formation assay from haematopoietic yolk sac precursors

Yolk sac precursors were isolated from E9.5 and E10.5 $sek1^{+/-}$, $sek1^{+/-}$ and $sek1^{-/-}$ embryos and the formation of erythroid, myeloid and mixed (erythroid and myeloid) colonies determined as described (Shalaby et al., 1995). Briefly, total yolk sac precursors were plated in IMDM methylcellulose containing 15% fetal bovine serum. The following cytokines were added: Erythropoietin (2 U/ml), IL-1α (650 U/ml), Insulin (10 μg/ml), IL-3 (100 U/ml), IL-11 (25 ng/ml) and KLCM (100 ng/ml). The sizes and numbers of colonies were counted 7 and 10 days after the start of culturing.

In vitro differentiation of B cells, macrophages/monocytes, and mast cells from embryonic precursors

Yolk sac and splanchnopleura precursor cells were plated at 2000 cells/well with irradiated S17 stromal cells and IL-7. After 3 days of expansion, cells were further differentiated into (1) mast cells using IL-3, (2) macrophages/monocytes using CSF1 and (3) B lymphocytes using S17 stromal cells and IL-7. Cytokine concentrations were as described (Cumano et al., 1993; Ray et al., 1996). Supernatants from B cell cultures were tested for secreted IgM after 8 days of culture using an IgM-specific ELISA. Three different E10.5 litters [total numbers: $sek1^{+/+}$ (n=5), $sek1^{+/-}$ (n=14) and $sek1^{-/-}$ (n=8)] were analysed with similar results (not shown).

Radiation chimeras

For generation of chimeric mice, livers were harvested from E11.5 $Ly9.1^{+}$ embryos. Single-cell suspensions of total liver cells (5×10^6 cells/0.5 ml) were injected into the tail vein of $Ly9.2^{+}$ 12-week-old recombination-deficient $rag^{-/-}$ host mice which had been irradiated (400 rads). Radiation chimeras were maintained under pathogen-free conditions. The development of T cells and B cells from liver precursors was determined at 2 and 3 months after transfer using lineage-specific antibodies. Single-cell suspensions of thymocytes, spleen cells and mesenteric lymph node cells from $sek1^{-/-}\rightarrow rag^{-/-}$ chimeras, $sek1^{+/-}\rightarrow rag^{-/-}$ chimeras and control $rag^{-/-}$ mice were stained with directly labelled Abs specific for cells of the T cell lineage (anti-CD4, anti-CD8α, anti-CD3ε, anti-TCRαβ, anti-CD69, anti-CD25, anti-HSA) and B cell lineage (anti-B220, anti-CD19, anti-CD25, anti-CD43, anti-IgM, anti-IgD, anti-CD23). All Abs were from PharMingen. Samples were analyzed using a FACScan (Becton Dickinson).

RESULTS

Sek1-mutation is lethal between E10.5 and E12.5

To elucidate the role of SEK1 in embryogenesis and non-lymphoid cell lineages, we generated *sek1* null mice. Mice heterozygous for the *sek1* mutation appear normal, are fertile and show no obvious defects in any organs. However, no homozygous *sek1* mutant mice were found among 106 newborn animals from intercrosses between heterozygous mice (Table 1). The ratio of wild-type to heterozygous offspring was 1:1.9, indicating a recessive lethal phenotype. These results confirm a previous study in which a *sek1* null mutation was reported to be embryonic lethal but the cause and time point of lethality were not analysed (Yang et al., 1997).

To determine the gestational time point of the *sek1* lethality, we examined embryos from heterozygous intercrosses at different developmental stages. $Sek1^{-/-}$ embryos died (determined as the time at which their hearts stopped beating) between E10.5 and E12.5 (Table 1). All $sek1^{-/-}$ embryos were severely anemic but were otherwise normal in appearance and overall morphology (Fig. 1A,B). At E13.5, all recovered $sek1^{-/-}$ embryos were dead and appeared to be at different stages of resorption. The lethal phenotype and the time point of death of homozygous *sek1* mutant embryos were identical in mouse lines derived from two independent ES cell clones. To confirm that SEK1 was deleted, SEK1 protein expression was analysed in ES cells and E8.5, 9.5 and 10.5 embryos by western blotting. The results showed expression of SEK1 protein in wild-type mice at all developmental stages analysed (Fig. 2), but that SEK1 was not expressed in $sek1^{-/-}$ mutant ES cells or embryos (Fig. 2). SEK1 mRNA was absent as judged by RT-PCR and northern blotting. Moreover, we could not detect any aberrant splicing using three different RT-PCR primer pairs spanning exon sequences located 5′ and 3′ to the disrupted locus (not shown).

Table 1. Genotypic and phenotypic analysis of neonates and embryos from $sek1^{+/-}$ intercrosses

Embryonic stage	Number of litters	Numbers of each *sek1* genotype		
		$sek1^{+/+}$	$sek1^{+/-}$	$sek1^{-/-}$
E8.5	4	5	15	8
E9.5	7	17	28	14
E10.5	13	30	65	22 (3 dead)
E11.5	13	23	45	19 (7 dead)
E12.5	17	33	67	16 (14 dead)
E13.5	7	10	22	8 (all dead)
E14.5	7	17	38	6 (all dead)
E15.5	2	3	10	0
Neonate	18	30	58	0

Embryos were isolated at the indicated time points of gestation and, within 24 hours after birth (neonate), analysed for viability and processed for histological and immunocytometric staining. Genotypes of embryos were determined by PCR. Dead embryos were defined as embryos in which the heart stopped beating. Beating hearts were monitored using inverted microscopy.

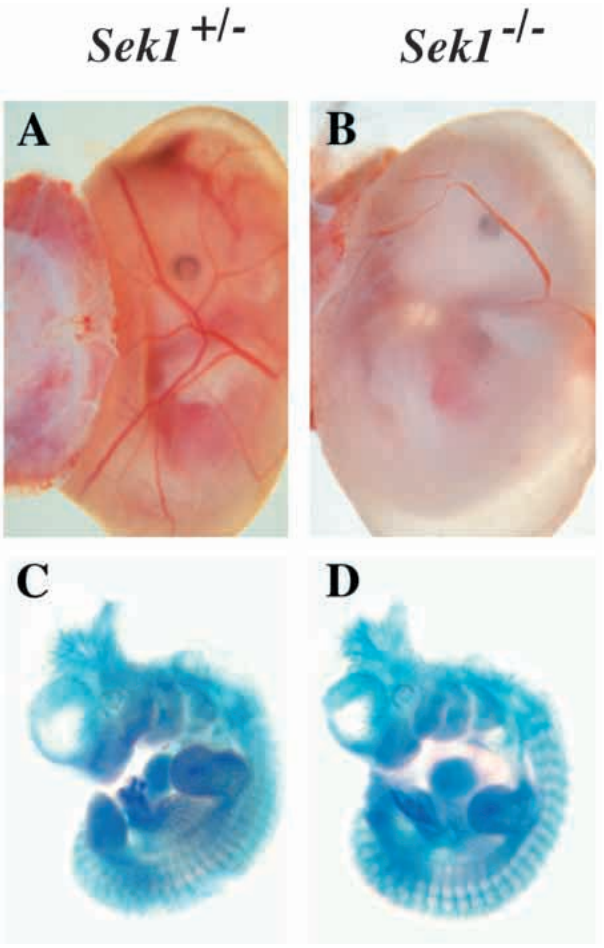

Fig. 1. Macroscopic appearance and vasculogenesis of *sek1*$^{-/-}$ and *sek1*$^{+/-}$ embryos. (A-D) E12.5 *sek1*$^{+/-}$ (A,C) and E12.5 *sek1*$^{-/-}$ (B,D) littermate embryos are shown before (A,B) and after (C,D) removal of chorioallantoic membranes and the placenta. Note the severe anemia in *sek1*$^{-/-}$ embryos. Placentae of *sek1*$^{-/-}$ embryos were macroscopically and histologically normal (not shown).

Normal vasculogenesis in *sek1*$^{-/-}$ embryos

Severe anemia was a consistent feature of the phenotype of *sek1*$^{-/-}$ embryos. Anemia was first observed at E9.5 (in 3 of 14 embryos) and some mutant embryos (3 of 22) died at E10.5 (Table 1). At E11.5, all *sek1*$^{-/-}$ embryos were severely anemic (not shown). To examine potential cellular mechanisms that could cause anemia and embryonic lethality, we analysed development of the vascular system, haematopoiesis and hepatogenesis in *sek1*$^{-/-}$ embryos.

To visualise the vascular system, we crossed a knock-in mutation of the *lacZ* gene into the receptor tyrosine kinase gene *flk1* to a *sek1* null background (Fong et al., 1995; Shalaby et al., 1995; Yamaguchi et al., 1993). In *flk1* heterozygous mice, expression of *lacZ* is regulated by the promoter of the *flk1* gene and is restricted to endothelial cells and their embryonic precursors. Embryos homozygous for *flk1* gene die between E8.5-9.5 as a result of an early defect in the development of haematopoietic (blood island formation) and endothelial cells (vasculogenesis) (Fong et al., 1995; Shalaby et al., 1995; Yamaguchi et al., 1993). *Sek1*$^{+/+}$*flk1*$^{+/-}$, *sek1*$^{+/-}$*flk1*$^{+/-}$ and *sek1*$^{-/-}$*flk1*$^{+/-}$ mice with flk1 promoter-regulated *lacZ* expression were generated from intercrosses between *sek1*$^{+/-}$ *flk1*$^{+/-}$ mice. All *Sek1*$^{-/-}$ *flk1*$^{+/-}$ embryos (12 of 12) were alive at E10.5 (not shown). Yolk-sac blood islands (not shown) and blood vessel development (Fig. 1C,D; and data not shown) were similar among *sek1*$^{-/-}$*flk1*$^{+/-}$, *sek1*$^{+/-}$*flk1*$^{+/-}$ and *sek1*$^{+/+}$*flk1*$^{+/-}$ embryos at E8.5, E9.5, E10.5, E11.5 and E12.5. These results show that SEK1 expression is not essential for yolk-sac blood-island formation and vasculogenesis during murine embryogenesis.

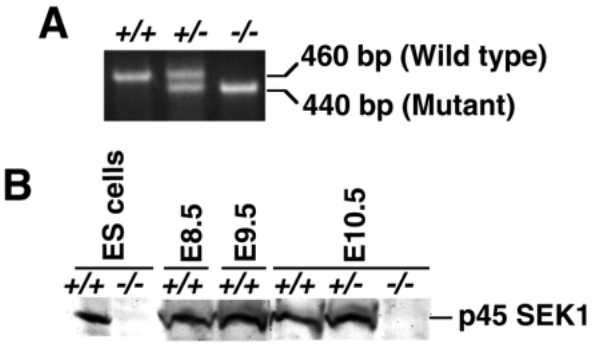

Fig. 2. Genotype identification and SEK1 expression in ES cells and embryos derived from heterozygous intercrosses. (A) PCR analysis of DNA isolated from embryos at 10.5 days of gestation. Mutant (440 bp) and wild type (460 bp) bands are indicated. (B) Western blot analysis of SEK1 protein (45 kDa) expression in ES cells and embryos at 8.5, 9.5 and 10.5 days of gestation. SAPK expression was comparable among *sek1*$^{+/+}$, *sek1*$^{+/-}$ and *sek1*$^{-/-}$ ES cells and embryos (not shown).

Normal haematopoiesis

A critical genetic checkpoint in embryogenesis is the switch from yolk sac- and splanchnopleura-dependent blood formation to liver-dependent haematopoiesis (Dieterlen Lievre et al., 1995; Godin et al., 1995). This switch in haematopoietic organs occurs around E10.5 to E12.5, precisely at the stage of development at which *sek1*$^{-/-}$ embryos die. To test whether the severe anemia and the lethal phenotype of *sek1* null embryos was due to an intrinsic defect in haematopoiesis, we examined erythroid, myeloid and mixed colony formation from E9.5 (Table 2) and E10.5 (not shown) yolk sac precursors using methylcellulose colony assays (Shalaby et al., 1995). The numbers (Table 2) and sizes (not shown) of erythroid, myeloid, mixed and total colonies were similar among *sek1*$^{-/-}$, *sek1*$^{+/-}$ and *sek1*$^{+/+}$ embryos. *Sek1*$^{-/-}$ and *sek1*$^{+/-}$ E9.5 and E10.5

Table 2. In vitro haematopoiesis from yolk sac precursors

	Number of colonies (±s.d.)			
Genotype	Erythroid	Myeloid	Mixed	Total
Sek1$^{+/+}$ (*n*=3)	89.3±20.9	508.3±63.1	42.7±16.6	640.3±72.8
Sek1$^{+/-}$ (*n*=5)	81.4±13.6	568.8±47.3	27.0±9.7	677.2±60.8
Sek1$^{-/-}$ (*n*=3)	79.0±31.0	696.5±126.5	25.5±16.5	800.5±174.5

Yolk sacs from E9.5 embryos were isolated and cultured in soft agar with a cocktail of different growth factors as described in Materials and Methods. Erythroid, myeloid and mixed colonies were counted 7 days after start of in vitro cutures. Similar results, i.e., no significant differences in the generation of erythroid, myeloid and mixed (erythroid and myeloid) colonies, were obtained at day 10 of in vitro cultures (not shown). s.d., standard deviation of the mean.

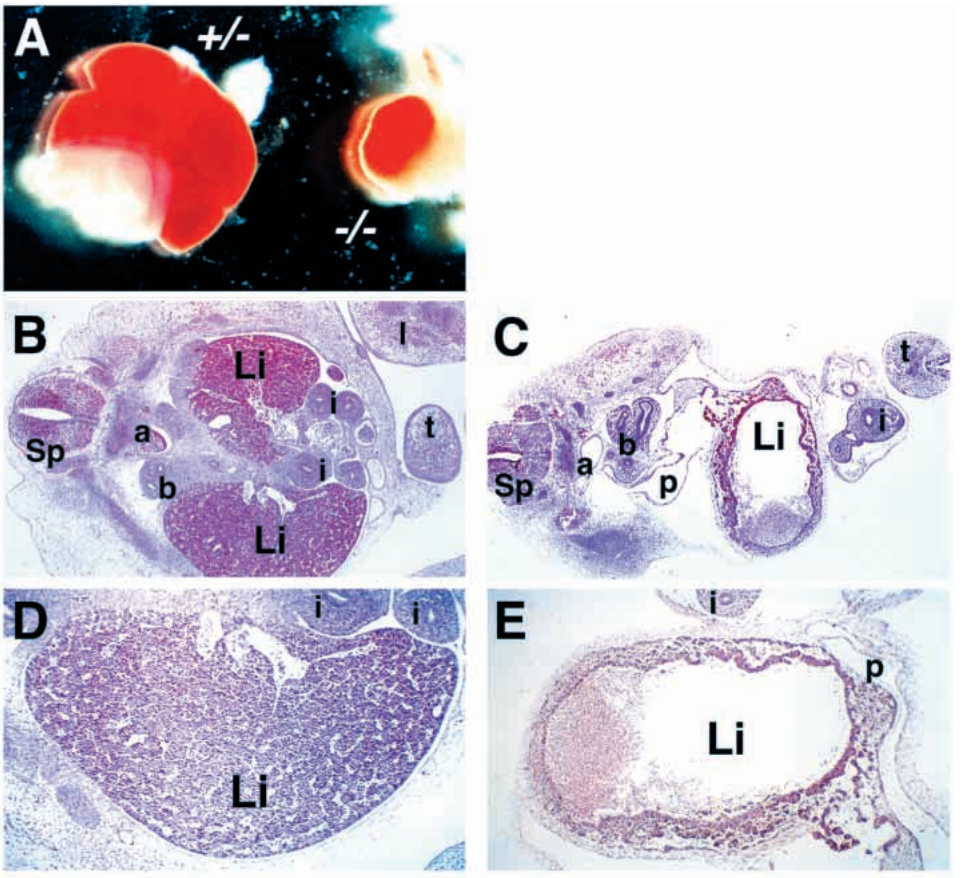

Fig. 3. Defective liver formation in *sek1*$^{-/-}$ embryos. (A) Macroscopic appearance of livers of E12.5 *sek1*$^{+/-}$ and *sek1*$^{-/-}$ littermates. (B-E) Transverse sections of E12.5 *sek1*$^{+/-}$ (B,D) and *sek1*$^{-/-}$ (C,E) littermate embryos. Note the absence of any parenchymal hepatocytes and residual haematopoietic cells in liver remnants of *sek1*$^{-/-}$ embryos. Li, liver; a, aorta; Sp, spinal cord; i, intestine; b, bronchi and lung buds; t, tail; l, limb; p, peritoneal cavity. HE stainings. (A) Magnification ×10; (B,C) magnification ×16; (D,E) magnification ×40.

embryos had comparable total numbers of haematopoietic precursors within the yolk sac and the splanchnopleura (not shown). Moreover, using a cocktail of different cytokines and stromal cell lines in a clonal in vitro differentiation assay (Cumano et al., 1993; Ray et al., 1996), *sek1*$^{-/-}$ and *sek1*$^{+/-}$ precursors derived from the yolk sac and the splanchnopleura of E10.5 embryos were stimulated to develop into macrophages/monocytes, mast cells (not shown), and immunoglobulin-secreting B lymphocytes (*sek1*$^{+/+}$ IgM [μg/ml]: 7.1±0.4 s.e.m., n=3; *sek1*$^{+/-}$ IgM [μg/ml]: 7.6±0.6, n=3; *sek1*$^{-/-}$ IgM [μg/ml]: 6.4±0.5, n=4; differences are not statistically significant, P>0.05). These results indicate that haematopoiesis from yolk sac- and splanchnopleura-derived precursors is not impaired in *sek1*$^{-/-}$ embryos.

Sek1$^{-/-}$ embryos have a defect in liver formation

Liver haematopoiesis and liver formation are crucial genetic checkpoints in embryogenesis (Dzierzak and Medvinsky,

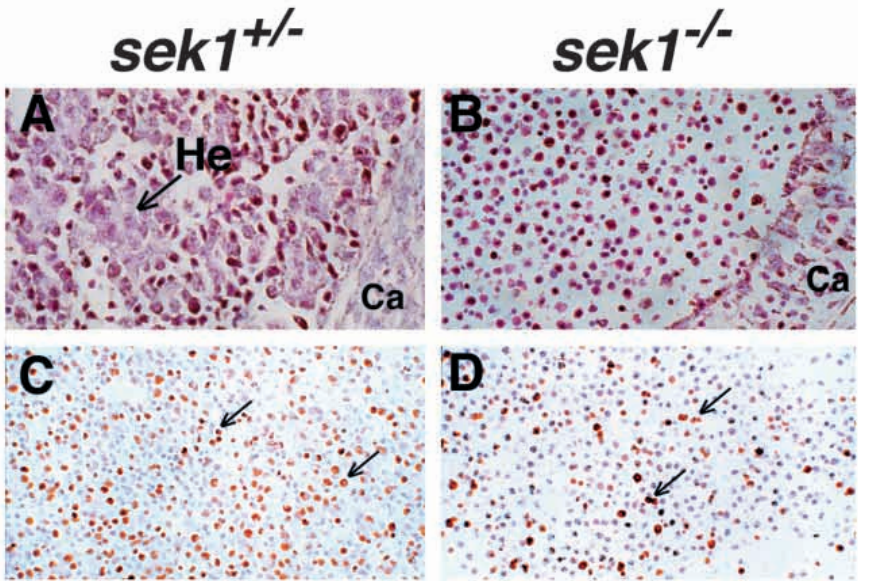

Fig. 4. Defective liver formation but normal proliferation of hematopoietic precursors in *sek1*$^{-/-}$ embryos. (A,B) Histological analysis of livers from E12.5 *sek1*$^{+/-}$ (A) and E12.5 *sek1*$^{-/-}$ (B) littermate embryos. Note the absence of parenchymal hepatocytes (He) in *sek1*$^{-/-}$ liver remnants. Ca, liver capsule. Transverse sections. HE staining. Magnification ×320. (C,D) PCNA immunostaining to detect proliferating cells in livers of E12.5 *sek1*$^{+/-}$ (C) and E12.5 *sek1*$^{-/-}$ (D) littermate embryos. Transverse cryosections were immunostained using anti-PCNA Ab. Horseradish peroxidase-labelled secondary Abs were used to detect anti-PCNA binding which appeared as a brown precipitate (arrows). Magnification ×160.

1995; Orkin and Zon, 1997). Whereas haematopoiesis (as judged by colony formation from the yolk sac and the growth of mast cells, B cells and macrophages/monocytes) and vasculogenesis (as assessed by the results from *sek1*$^{-/-}$*flk1*$^{+/-}$-*lacZ* mice) appear normal in *sek1*$^{-/-}$ mice, these mutants had very small livers (Fig. 3A). Histological analysis revealed that livers from E11.5 (not shown) and E12.5 *sek1*$^{-/-}$ embryos were severely disorganized and contained significantly reduced numbers of parenchymal hepatocytes (Fig. 3B-E, Fig. 4A,B). Anti-cytokeratin immunostaining (Hilberg et al., 1993; Johnson et al., 1993) showed that few disorganized islands of cytokeratin-expressing hepatocytes were still present in liver remnants of E11.5 and E12.5 *sek1*$^{-/-}$ embryos (Fig. 9F, and data not shown). Importantly, liver remnants from *sek1*$^{-/-}$ embryos contained haematopoietic precursor cells (Fig. 4A,B) and large clusters of erythroid cells indicative of hepatic erythropoiesis (not shown). These haematopoietic precursor cells stained positive for the proliferating cell nuclear antigen (PCNA) marker implying that these cells were indeed proliferating (Fig. 4C,D). Moreover, transfer of haematopoietic precursors from E11.5 *sek1*$^{-/-}$ liver remnants into adult irradiated *rag*$^{-/-}$ host animals revealed that the *sek1*$^{-/-}$ haematopoietic precursors could develop into phenotypically normal thymocyte populations, mature lymph node CD4$^+$ and CD8$^+$ T cells and splenic sIgM$^+$ and sIgD$^+$ B cells at a frequency and efficiency similar to haematopoietic precursors isolated from E11.5 *sek1*$^{+/-}$ livers (Fig. 5). In addition, T and B cells from these *sek1*$^{-/-}$*rag*$^{-/-}$ chimeras were functionally competent in terms of antigen driven-proliferation and cytokine production (not shown). Thus, haematopoietic precursors present in *sek1*$^{-/-}$ liver remnants are functional and can give rise to various lymphoid cell lineages in a *rag*$^{-/-}$ environment in vivo. Histological analysis of all other organs did not reveal any defects. These data show that SEK1 specifically regulates liver formation and, in particular, the development of hepatocytes.

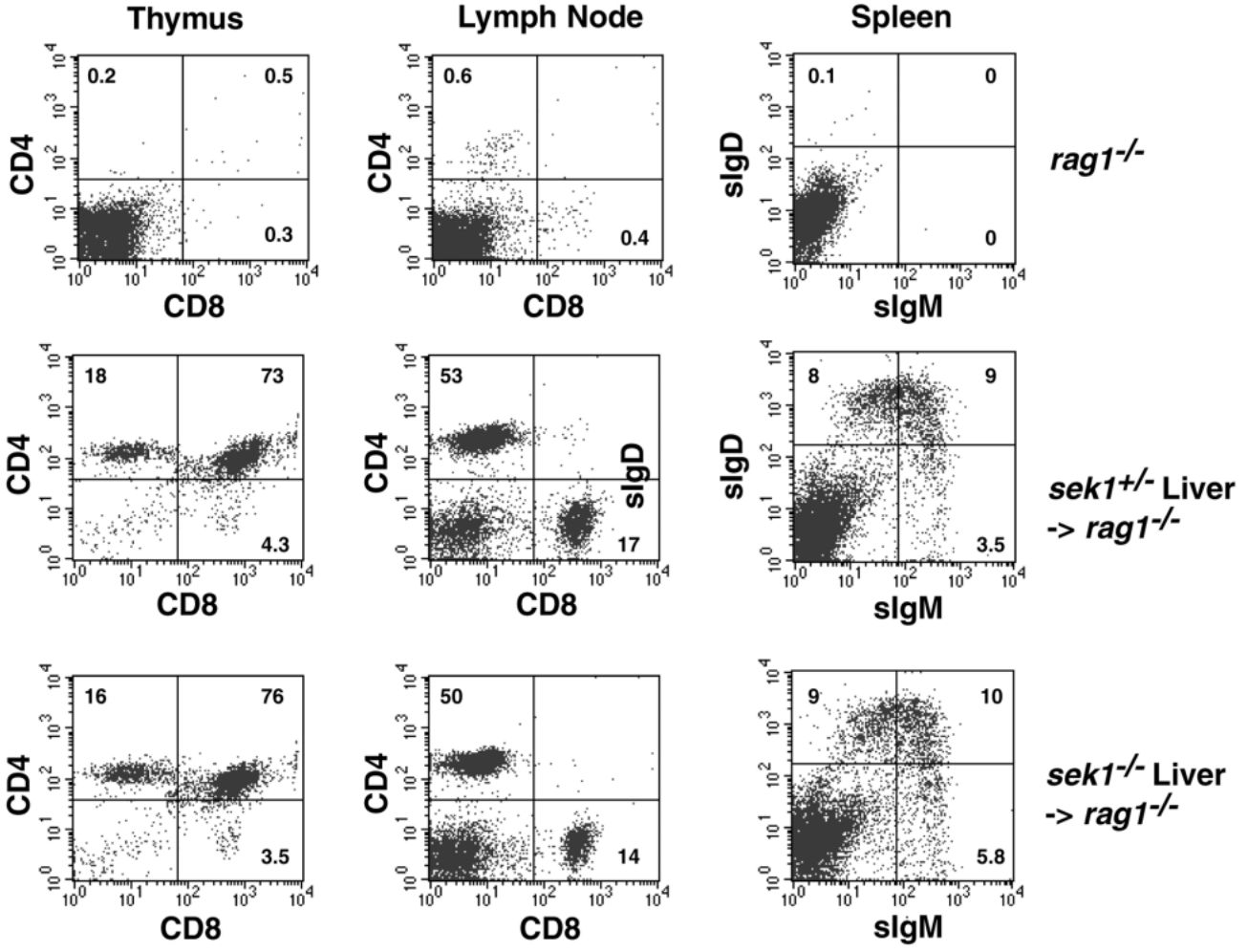

Fig. 5. Fetal liver cells transfer. Fetal liver cells (5×10^6 cells/0.5 ml) from E11.5 *sek1*$^{+/-}$ and E11.5 *sek1*$^{-/-}$ embryos were transferred into *rag1*$^{-/-}$ hosts as described in Materials and methods. Thymocytes, total lymph node cells, and total splenocytes were isolated from *sek1*$^{-/-}$→*rag*$^{-/-}$ and *sek1*$^{+/-}$→*rag*$^{-/-}$ chimeras 3 months after after transfer and control *rag*$^{-/-}$ mice and stained with lineage-specific antibodies to determine thymocyte maturation (CD4 versus CD8, left panels), mature lymph node T cell populations (CD4 versus CD8, middle panels), and mature splenic B cells (sIgM versus sIgD, right panels). It should be noted that expression levels of TCRαβ, CD69, CD25 and HSA on thymocytes and peripheral lymph node T cells and the expression levels of B220, CD19, CD25 and CD23 on splenic B cells were comparable among *sek1*$^{-/-}$*rag*$^{-/-}$ and *sek1*$^{+/-}$*rag*$^{-/-}$ chimeric mice. Percentages of positive cells within a quadrant are indicated. One result representative of 3 experiments is shown.

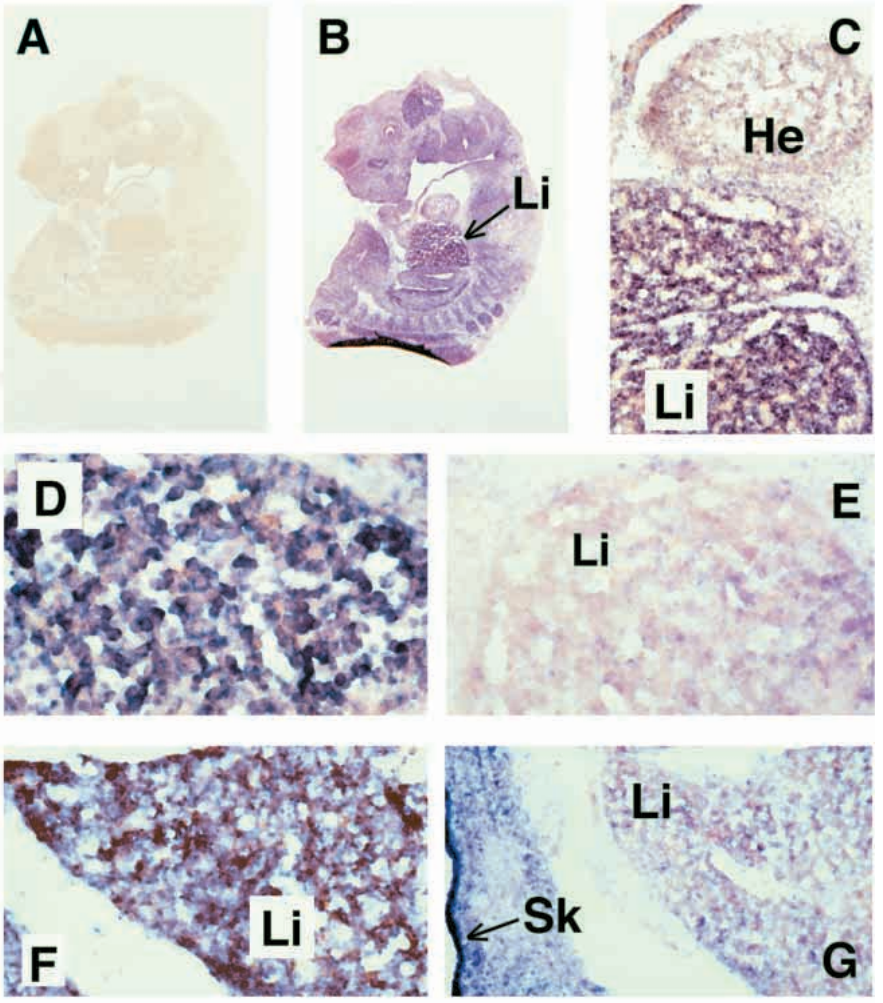

Fig. 6. Tissue distribution of sek1 and sek2 mRNA in embryos detected by in situ hybridization. (A-C) Distribution of sek1 mRNA in E10.5 embryos as detected by a full-length sek1 antisense probe (B,C). Li, liver; He, heart. A sek1-sense probe was used as a negative control for in situ hybridization (A). Note the ubiquitous and high level of expression of sek1 mRNA in all organs. Similar expression patterns can be observed in E12.5, E14.5 and E16.5 embryos (not shown). Magnifications: A,B ×6; C ×80. (D-G) Expression of sek1 (D,F) and sek2 (E,G) mRNA in livers of E12.5 (D,E) and E14.5 (F,G) embryos. Sek1 mRNA is very highly expressed in the liver (Li) compared to sek2 mRNA. Note the high level of expression of sek2 mRNA in the skin (Sk). Magnifications: D,E ×320; F,G ×160.

Localization of SEK1 expression

Since the phenotypic defect of the *sek1* null mutation was restricted to the liver, we analysed SEK1 expression by in situ hybridization. SEK1 mRNA is highly expressed in all embryonic tissues of E10.5 (Fig. 6A-C), E12.5, E14.5 and E16.5 embryos (not shown). Although SEK2/MKK7 is also ubiquitously expressed in all organs, the level of expression of SEK2 mRNA is lower than that of SEK1 mRNA, and SEK2 is found mainly in epithelial tissues such as skin, lung epithelium and epithelial layers lining the olfactory cavity and developing teeth (not shown) (Yao et al., 1997). Interestingly, whereas SEK1 mRNA expression was very high in livers of E12.5 and E14.5 embryos (Fig. 6D,F), liver expression of SEK2 was found to be very low (Fig. 6E,G). In fact, among all organs analysed by in situ hybridization SEK2 mRNA expression was lowest in the liver and the heart (not shown).

Sek1$^{-/-}$ embryos can form a primordial liver

To determine the molecular mechanism underlying the lack of parenchymal hepatocytes, we analysed liver formation in E7.5, E8.5, E9.5, E10.5 and E11.5 embryos. To test whether the hepatocyte defect was due to a defect in visceral endoderm development (from which hepatocytes are derived), we performed whole-mount immunostaining for hepatocyte nuclear factor 3β (HNF3β), a transcription factor present in the embryonic notochord floorplate, visceral endoderm, hepatocyte precursors and mature hepatocytes (Gualdi et al., 1996; Marti et al., 1995; Sladek, 1993). HNF3β staining of E8.5 and E9.5 *sek1*$^{+/+}$, *sek1*$^{+/-}$ and *sek1*$^{-/-}$ embryos showed that the visceral endoderm and the liver anlage form normally in the absence of SEK1 (Fig. 7; and data not shown). Moreover, cytokeratin-positive parenchymal liver cells were present in histological sections of E9.5 (not shown) and E10.5 *sek1*$^{-/-}$ embryos (Figs 8A-D, 9A,B). However, at E10.5, *sek1*$^{-/-}$ livers were found to be at various stages of disorganization, suggesting variable penetrance of the mutation in E10.5 embryos (Fig. 8C,D, and data not shown). Liver cells gradually disappeared and at E12.5 only small and disorganized islands of parenchymal liver cells were detectable (Fig. 8G). These parenchymal islands are hepatocytes as determined by cytokeratin immunostaining (Fig. 9F). These results indicate that the liver anlage forms normally in *sek1*$^{-/-}$ embryos but that parenchymal hepatocytes disappear from the liver anlage starting from E10.5 of embryogenesis.

Massive liver cell apoptosis in *sek1*$^{-/-}$ embryos

SEK1-regulated SAPK activation has been implicated both in the induction (Chen et al., 1996a; Li et al., 1996; Verheij et al.,

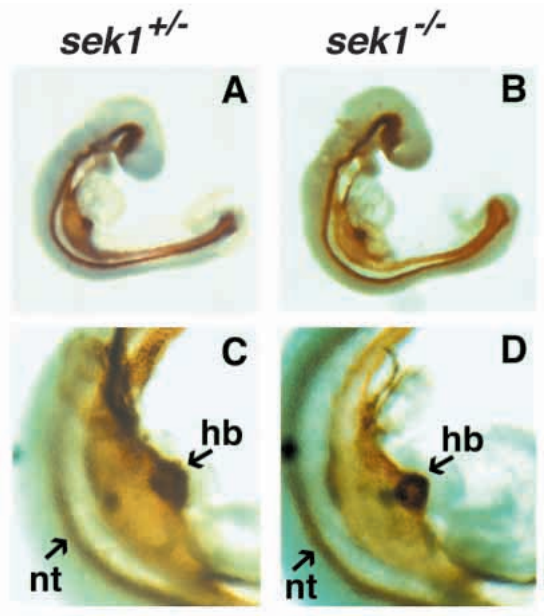

Fig. 7. Formation of the primordial liver. Whole-body HNF3β staining of E9.5 *sek1*$^{+/-}$ (A,C) and E9.5 *sek1*$^{-/-}$ (B,D) littermates. Embryos were isolated at E9.5 and HNF3β expression in the notochord and floorplate (nt), the visceral endoderm and the hepatic bud (hb) were determined as described in Materials and Methods. C and D are enhanced images of A and B. Magnification: A,B ×4; C,D ×16.

1996; Westwick et al., 1995; D. Yang et al., 1997a) and suppression of apoptosis in response to developmental death signals (Nishina et al., 1997). We therefore analysed apoptosis in liver cells using in situ Tunel staining. Apoptosis was infrequent in livers from E10.5 *sek1*$^{-/-}$ and littermate E10.5 *sek1*$^{+/-}$ embryos as determined by Tunel staining (Fig. 9A-D). However, in some cases, increased apoptosis of liver cells was already detectable in E10.5 *sek1*$^{-/-}$ embryos depending on the variable penetrance of liver disorganization. Importantly, on E12.5 livers of all *sek1*$^{-/-}$ embryos analyzed showed areas of massive apoptosis. These apoptotic cells were confined to residual islands of cytokeratin-positive hepatocytes (Fig. 9E-H). Increased apoptosis was not apparent in haematopoietic precursors present in liver remnants of E12.5 *sek1*$^{-/-}$ embryos (not shown), suggesting that the enhanced apoptosis was restricted to parenchymal hepatocytes. The numbers of apoptotic cells in all other organs such as the developing limb bud, heart or the brain were comparable between *sek1*$^{-/-}$, *sek1*$^{+/-}$ and *sek1*$^{+/+}$ embryos at E10.5, E11.5 and E12.5 (not shown). These results imply that SEK1 and SEK1-regulated signaling are essential for hepatocyte survival during early liver formation.

DISCUSSION

SAPKs, which are expressed in all organs, are activated in response to a plethora of environmental and developmental cues. Our finding that SEK1 null mice have a defect confined to hepatocytes indicates that specific SAPK activation is important during embryogenesis and morphogenesis. These results provide the first genetic link between stress-responsive kinases and organogenesis in mammals. Moreover, these results suggest that environmental toxins that influence SAPK function could lead to embryonic tissue-specific malformation and developmental defects.

Liver formation is a particularly crucial genetic check point in fetal haematopoiesis and fetal development. In *sek1*$^{-/-}$ mice, liver formation as determined by HNF3β staining was normal until E9.5 with the first histological changes appearing at E10.5 of embryogenesis. Livers from E11.5 and E12.5 *sek1*$^{-/-}$ embryos were very small and these liver remnants contained a capsule, haematopoietic precursors, and disorganized islands of parenchymal hepatocytes. Mechanistically, liver cells from *sek1*$^{-/-}$ mice underwent a massive wave of apoptosis,

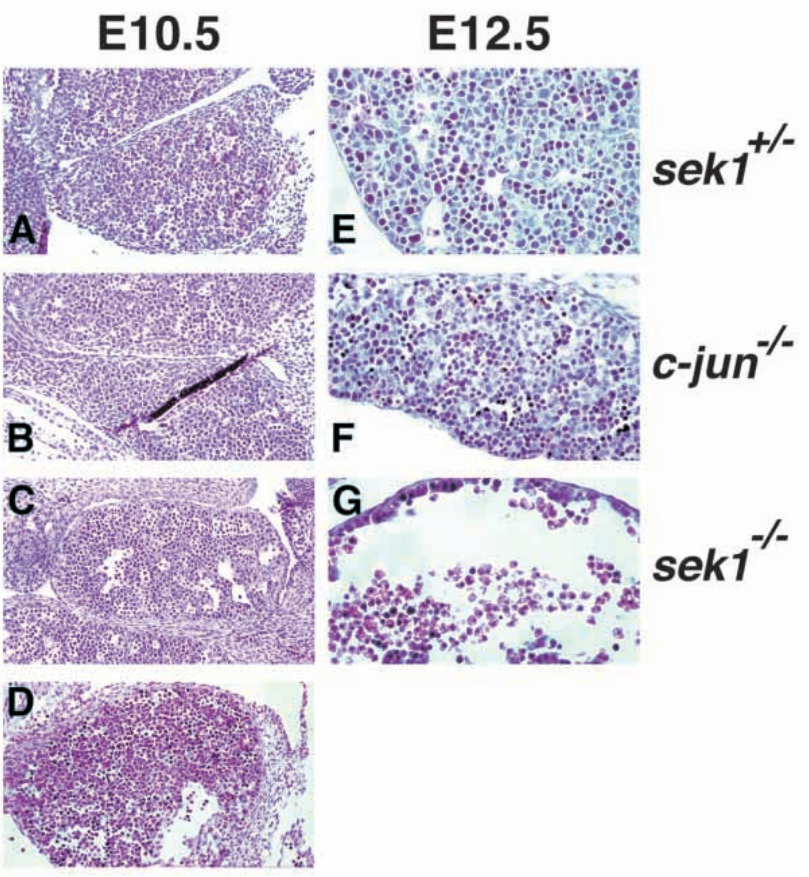

Fig. 8. Liver formation in *sek1*$^{-/-}$ and *c-jun*$^{-/-}$ embryos. (A-D) Livers of E10.5 *sek1*$^{+/-}$ (A), E10.5 *c-jun*$^{-/-}$ (B), and E10.5 *sek1*$^{-/-}$ (C,D) embryos. Note the variable penetrance of the liver formation defect in E10.5 *sek1*$^{-/-}$ embryos (compare C and D). Longitudinal sections. HE staining. Magnifications: A-D ×80. (E-G) Livers of E12.5 *sek1*$^{+/-}$ (E), E12.5 *c-jun*$^{-/-}$ (F) and E12.5 *sek1*$^{-/-}$ (G) embryos. At E11.5 (not shown) and E12.5, hepatocyte numbers were significantly reduced in all *sek1*$^{-/-}$ embryos analysed (not shown). Longitudinal sections. HE staining. Magnifications: E-G ×160.

suggesting that SEK1 provides a survival signal for hepatocytes during a defined stage of liver morphogenesis. These findings raise two important questions: What receptors trigger SEK1 activation and hepatocyte survival during embryogenesis, and why is the defect limited to hepatocytes despite the fact that SEK1 is ubiquitously expressed?

The activation of SAPK/JNK signalling cascades occurs not only in response to many types of cellular stresses but also in response to growth factors, heterotrimeric G-proteins, phorbol esters and co-stimulatory activation of T lymphocytes (Darby et al., 1994; Derijard et al., 1994; Minden et al., 1994a,b; Prasad et al., 1995; Raingeaud et al., 1996; Su et al., 1994). Various growth factors such as Scatter factor (SF)/Hepatocyte growth factor (HGF), EGF, IL1 and TNFα have been implicated in hepatogenesis and liver regeneration following partial hepatectomy (Diehl and Rai, 1996; Naldini et al., 1991). In particular, TNFα, IL1 and EGF lead to activation of SEK1 and the SAPK pathway (Kyriakis et al., 1994; Minden et al., 1994a,b). However, mice deficient for either the TNF, TNFR, IL-1 or IL-1R genes have no reported defects in liver formation (Mittrucker et al., 1996). By contrast, *hgf*$^{-/-}$ mice die around E13.5 to E16.5 of embryogenesis due to liver failure, and embryonic livers from these mice are reduced in size and show extensive loss of parenchymal cells (Schmidt et al., 1995; Uehara et al., 1995). It has been reported that HGF can provide survival signals for liver progenitor cells or epithelial cells via activation of the c-Met-encoded HGF receptor (Bardelli et al., 1996; Longati et al., 1996), and that activation of the SAPK pathway is essential for transformation by the chimeric Trp-Met oncogene in fibroblasts (Rodrigues et al., 1997). Thus, c-Met activation was a good candidate for relaying HGF stimulation to SAPK activation and promoting hepatocyte survival. However, we failed to detect any SAPK activation in cultured hepatocyte lines and primary fetal liver cells following HGF treatment (not shown), suggesting that c-Met activation per se does not lead to SAPK activity. Further, *hgf*$^{-/-}$ mice die later than *sek1*$^{-/-}$ mice and have an additional defect in placental development (Schmidt et al., 1995; Uehara et al., 1995). Nevertheless, the lack of HGF-mediated SAPK activation in vitro and the fact that HGF has differential effects during organogenesis do not exclude the possibility that HGF acts via SEK1. Various cytokines and cytokine receptors most likely cooperate during liver morphogenesis to provide the crucial hepatocyte survival signal. Alternatively, it may be possible that adhesion receptors on hepatocytes regulate cell survival via activation of the related adhesion focal tyrosine kinase (RAFTK or PYK2) (Ganju et al., 1998; Tokiwa et al., 1996), or the Rho-family G-proteins Rac1 and CDC42 (Bagrodia et al., 1995; Coso et al., 1995; Minden et al., 1995; Olson et al., 1995) which couple surface receptors to SAPK activation.

It has been suggested that the SEK1→SAPK/JNK→c-Jun signaling cascade is a common pathway required for the induction of apoptosis in response to many types of stress (Chen et al., 1996a,b; Westwick et al., 1995). For example, overexpression of dominant negative SEK1 can block the induction of cell death by heat shock, irradiation, anti-cancer drugs, peroxide, ceramide or cytokine deprivation (Cuvillier et al., 1996; Verheij et al., 1996; Xia et al., 1995; Zanke et al., 1996). Apoptosis is also induced by overexpression of the SAPK-activating MAPKKK homologue apoptosis signal-regulating kinase-1 (ASK1), which can mediate SEK1 and SAPK activation in response to TNFα (Ichijo et al., 1997). However, apoptosis does occur in *sek1*$^{-/-}$ ES cells, thymocytes and splenic T cells in response to anisomycin, serum depletion, UV- and γ-irradiation, osmolarity changes, heat shock, anti-cancer drugs, CD3/CD28 ligation or PMA/Ca^{2+}-ionophore, with similar kinetics and at similar doses as in *sek1*$^{+/+}$ and *sek1*$^{+/-}$ cells (Nishina et al., 1997). Similarly, apoptotic cell death can be observed in the absence of SAPK activation induced via TNF-Rp55 (Liu et al., 1996; Natoli et al., 1997) or Fas (Lenczowski et al., 1997). These results contradict the hypothesis that SEK1-mediated activation of SAPK/JNK is required for the induction of cell death in response to all apoptosis inducers. Rather, this pathway must operate in a signal-specific and perhaps cell-type-dependent fashion.

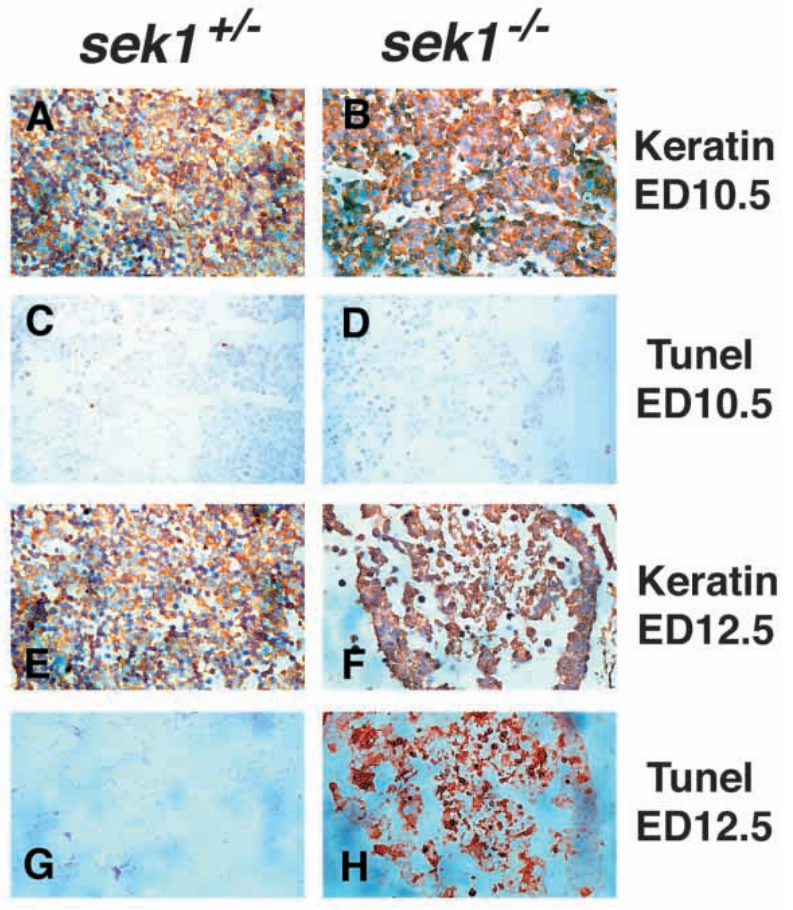

Fig. 9. Cytokeratin staining and detection of apoptosis in *sek1*$^{-/-}$ livers. Liver cryosections isolated from E10.5 *sek1*$^{+/-}$ (A,C), E10.5 *sek1*$^{-/-}$ (B,D), E12.5 *sek1*$^{+/-}$ (E,G), and E12.5 *sek1*$^{-/-}$ (F,H) littermates were analysed for for the presence of parenchymal hepatocytes using cytokeratin immunostaining (Keratin). Apoptosis was detected by TUNEL staining. All samples were counterstained with hematoxylin. Positive cells exhibit a brown precipitate. All magnification ×160.

Interestingly, SEK1 appears to provide a survival signal for hepatocytes during liver morphogenesis. Hypothetically, SEK1 signaling could induce anti-apoptotic genes in developing hepatocytes and/or directly influence signaling pathways required for cell survival. It is also possible that SEK1 has multiple functions in cell proliferation, survival, maturation and development, depending on the type of cell and its developmental stage.

SEK1-mediated activation of SAPKs/JNKs leads to phosphorylation of c-Jun and activation of Jun/Fos heterodimeric AP-1 transcriptional complexes, which are generally believed to be positive regulators of transcription (Angel and Karin, 1991; Kallunki et al., 1994; Kyriakis et al., 1994; Lin et al., 1995; Sanchez et al., 1994). The *Drosophila hemipterous* (*hep*) gene is a homolog of the mammalian SEK1/SEK2 family of dual specificity kinases (Holland et al., 1997; Yao et al., 1997). Hep controls the morphogenetic activity of the dorsal epidermis and epithelial cell sheet movement (Glise et al., 1995). Mutations of the *Drosophila* SAPK-homologue *basket* and the fly homologue of mammalian Jun, Djun, also lead to a defect in dorsal closure (Glise and Noselli, 1997; Kockel et al., 1997; Riesgo-Escovar et al., 1996; Sluss et al., 1996), implying that the Hep→Basket→DJun signaling cascade is essential for epithelial cell movement during fly morphogenesis. *Kayak* encodes the *Drosophila* homologue of the mammalian *c-fos* proto-oncogene product and *kayak* (DFos) mutant flies have a defect in dorsal closure, indicating that AP-1 transcriptional complexes are crucial for epithelial cell movement in *Drosophila* (Riese et al., 1997; Riesgo-Escovar and Hafen, 1997; Zeitlinger et al., 1997).

Mice bearing mutations of components of the stress signaling cascade have been previously reported. Mice lacking the brain-specific SAPKβ/JNK3 isoform undergo normal embryogenesis and appear normal (Yang et al., 1997) whereas *c-jun*$^{-/-}$ embryos display defective liver organization and die between E13.5 and E14.5 (Hilberg et al., 1993; Johnson et al., 1993). However, liver defects are much more severe in *sek1*$^{-/-}$ embryos than in *c-jun*$^{-/-}$ mice and livers from E12.5 *c-jun*$^{-/-}$ embryos contain residual hepatocytes (Fig. 8E-F). *C-jun*$^{-/-}$ liver cells display, albeit infrequently, morphological changes indicative of apoptosis (Hilberg et al., 1993). *C-fos*$^{-/-}$ mice are viable but have a defect in osteoclast differentiation and tooth eruption (Grigoriadis et al., 1994; Wang et al., 1992). Results form our laboratory show that *sek1*$^{-/-}$*c-jun*$^{-/-}$ double mutant mice died very early in embryogenesis between E7.5 and E8.5 (not shown). The cause of lethality in *sek1*$^{-/-}$*c-jun*$^{-/-}$ embryos is not yet known. Moreover, preliminary data indicate that c-Fos and SEK1 cooperate in liver formation and *sek1*$^{-/-}$*c-fos*$^{-/-}$ double mutant mice die earlier than *sek1*$^{-/-}$ single mutant mice (not shown). These results in *c-jun*$^{-/-}$*sek1*$^{-/-}$ and *sek1*$^{-/-}$*c-fos*$^{-/-}$ double mutant mice show that SEK1, c-Jun and c-Fos cooperate during mammalian embryogenesis. During liver formation, SEK1 probably activates transcriptional factors such as c-Jun and AP-1. Importantly, our results in *c-jun*$^{-/-}$ *sek1*$^{-/-}$ double mutant embryos indicate that SEK1 and c-Jun have an additional role in early embryonic development that is independent of hepatogenesis and liver cell survival.

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